EDGEWOOD CHEMICAL BIOLOGICAL CENTER
U.S. ARMY RESEARCH, DEVELOPMENT AND ENGINEERING COMMAND
Aberdeen Proving Ground, MD 21010-5424

ECBC-TR-1347

BIODEGRADATION OF ORGANOFLUORINE COMPOUNDS

Melissa M. Dixon
Steve P. Harvey

RESEARCH AND TECHNOLOGY DIRECTORATE

Jennifer E. Mohr

MINORITY UNDERGRADUATE STUDENT INTERNSHIP PROGRAM/
OAK RIDGE INSTITUTE FOR SCIENCE AND EDUCATION
Belcamp, MD 21017-1432

February 2016

Disclaimer

The findings in this report are not to be construed as an official Department of the Army position unless so designated by other authorizing documents.

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE *(DD-MM-YYYY)*	**2. REPORT TYPE**	**3. DATES COVERED** *(From - To)*
XX-02-2016	Final	Mar 2011 – Sep 2011

4. TITLE AND SUBTITLE
Biodegradation of Organofluorine Compounds

5a. CONTRACT NUMBER

5b. GRANT NUMBER

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
Dixon, Melissa M.; Harvey, Steve P. (ECBC); Mohr, Jennifer E. (MUSIP/ORISE)

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Director, ECBC, ATTN: RDCB-DRB-C, APG, MD 21010-5424
Minority Undergraduate Student Internship Program/Oak Ridge Institute for Science and Education, 4692 Millennium Drive, Suite 101, Belcamp, MD 21017-1432

8. PERFORMING ORGANIZATION REPORT NUMBER
ECBC-TR-1347

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Edgewood Chemical Biological Center Seedling Program

10. SPONSOR/MONITOR'S ACRONYM(S)
ECBC Seedling Program

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for public release; distribution is unlimited.

13. SUPPLEMENTARY NOTES

14. ABSTRACT:

Enrichment cultures were set up using various organofluorine compounds as sole carbon sources for growth, which was confirmed on two substrates: benzoyl fluoride and 1*H*,1*H*,2*H*,2*H*-perfluorooctyltriethoxysilane. Subsequent measurements of defluorination rates showed that benzoyl fluoride defluorinated very rapidly; therefore, the growth observed was probably on benzoic acid, a hydrolysis product. Both cultures appeared to be consortia; single-colony isolates capable of growth on either compound have not yet been identified. A continuous-loop UV mutagenesis reactor was set up to create segregates with newly derived abilities to use organofluorine compounds as sole carbon sources. This work will be continued in future studies.

15. SUBJECT TERMS
Organofluorine Biodegradation Defluorination Hydrolysis

16. SECURITY CLASSIFICATION OF:			**17. LIMITATION OF ABSTRACT**	**18. NUMBER OF PAGES**	**19a. NAME OF RESPONSIBLE PERSON**
a. REPORT	**b. ABSTRACT**	**c. THIS PAGE**			**19b. TELEPHONE NUMBER** *(include area code)*
U	U	U	UU	18	Renu B. Rastogi (410) 436-7545

Blank

PREFACE

The work described in this report was authorized under the U.S. Army Edgewood Chemical Biological Center Seedling Program. This work was started in March 2011 and completed in September 2011.

The use of either trade or manufacturers' names in this report does not constitute an official endorsement of any commercial products. This report may not be cited for purposes of advertisement.

This report has been approved for public release.

Blank

CONTENTS

FIGURES

TABLES

SCHEME

BIODEGRADATION OF ORGANOFLUORINE COMPOUNDS

1. INTRODUCTION

Organofluorines consist of an important group of compounds that are used as refrigerants and agricultural chemicals. Although useful, organofluorines are frequently toxic and can cause environmentally deleterious effects such as ozone depletion.[1] The challenge associated with their remediation and detoxification is largely related to their stability. The carbon–fluorine (C–F) bond is the strongest covalent bond a carbon atom can form, and it typically survives extreme pH, temperature, and oxidation-reduction conditions.[2] New and improved means of detoxifying these compounds are highly sought after, and a few enzymes that are capable of cleaving the C–F bond have been identified.[3,4] Recently, several of these enzymes have been crystallized.[2] Other defluorinases exist, as evidenced by the fact that there are 12 known naturally occurring organofluorine compounds, all of which have been found in microorganisms and plants.[5] As with all compounds found in nature, it is assumed that their enzymatic degradation occurs because they have not accumulated infinitely in the environment.

2. MATERIALS AND METHODS

An M9 medium was used without glucose. It consisted of 7.25 g of Na_2HPO_4, 3.0 g of KH_2PO_4, 0.71 g of NaCl, and 2.0 g of NH_4Cl per liter. A salt solution was added to the following concentrations: $MgSO_4$ (1 mM), $CaCl_2$ (0.1 mM), and $FeCl_3$ (0.01 mM).

Fluoride electrode assays were conducted at room temperature in 2.5 mL of 50 mM bis-tris-propane (BTP) buffer with pH 7, using 100 μL of supernatant from the lysate as the enzyme sample.

3. RESULTS AND DISCUSSION

3.1 Enrichment Cultures

One approach to the identification of degradative enzymes is to “feed” the compound of interest (COI) to a consortium of microorganisms as the sole carbon source for growth and energy. This selection is a powerful tool that can be used to isolate organisms and, subsequently, enzymes for the degradation of various organic compounds. Organisms capable of COI use overgrow the culture and after a period of time (typically weeks), can be isolated from the consortium. Following isolation, lysates can be tested for useful enzyme activity; in the specific case of C–F hydrolysis, activity can be monitored by fluoride electrode. Our approach was to use an array of naturally occurring and synthetic organofluorine compounds as carbon sources in separate enrichments.

The compounds used in the enrichments are shown in Table 1. All compounds were initially used as a 20 mM concentration as a sole carbon source in an M9 salt medium.

Table 1. Compounds Used as Carbon Sources in Enrichments

Compound	MW	Density g/mL @ 25 °C
Chloropentafluorobenzene	202.51	1.569
1*H*,1*H*,2*H*,2*H*-perfluorooctyltriethoxysilane	510.35	1.3299
2,2,2-Trifluoroethanol	100.04	1.373
4-Fluorophenol	112.1	1.246
Perfluorononanoic acid	464.08	Solid
Perfluoro-1-octanesulfonyl fluoride	502.12	1.824
2,2-Difluoroethanol	82.05	1.296
2-Fluroaniline	111.12	1.151
2,6-Dichloro-4-(trifluoromethyl) pyridine	215.99	1.505
Benzoyl fluoride	124.11	1.14
2-Fluoro-6-(trifluoromethyl) benzoyl chloride	226.56	1.465
5-Fluoro-2-nitrobenzotrifluoride	209.1	1.49
3-Chloro-4-fluoro-5-nitrobenzotrifluoride	243.54	1.607
2-Fluoro-3-(trifluoromethyl) benzoic acid	208.11	Solid
5-Fluoro-2-(trifluoromethyl) benzoic acid	208.11	Solid
2-Fluoro-5-nitrobenzotrifluoride	209.1	1.52
2-Fluoro-6-(trifluoromethyl) benzonitrile	189.11	1.373

Enrichment cultures were inoculated with environmental isolates from water, soil, and wastewater discharges and incubated at 25 °C for up to 3 months. Two of the enrichments (benzoyl fluoride and 1*H*,1*H*,2*H*,2*H*-perfluorooctyltriethoxysilane) became turbid as a result of bacterial growth. Initial cultures containing these two substrates were subsequently passed using a 1 µL inoculum placed into a 100 mL flask containing the M9 medium with 20 mM of the same compound as the sole carbon source. Both cultures continued to grow with several subsequent passages, and bacteria from each were repeatedly streaked for single colonies. Neither was capable of growth on either substrate as a sole carbon source.

Both cultures were grown in 1 L volumes to their maximum density on their respective substrates. They were then pelleted at 5,000 × g, lysed in a microfluidizer, and pelleted again at 20,000 × g. The supernatant was tested for defluorination activity using a fluoride electrode calibrated against known standards. Assays were run with all the compounds listed in Table 1 against lysates from cultures grown on benzoyl fluoride and 1*H*,1*H*,2*H*,2*H*-perfluorooctyltriethoxysilane. This screening did not identify any significant activity from either culture on the organofluorine compounds that were tested. Figure 1 depicts a typical result of an assay comparing defluorination rates in the presence and absence of bacterial lysates. In all the cases, rates were similar with or without the lysates, which was consistent with little or no enzymatic activity. Table 2 shows defluorination rates of some organofluorine compounds.

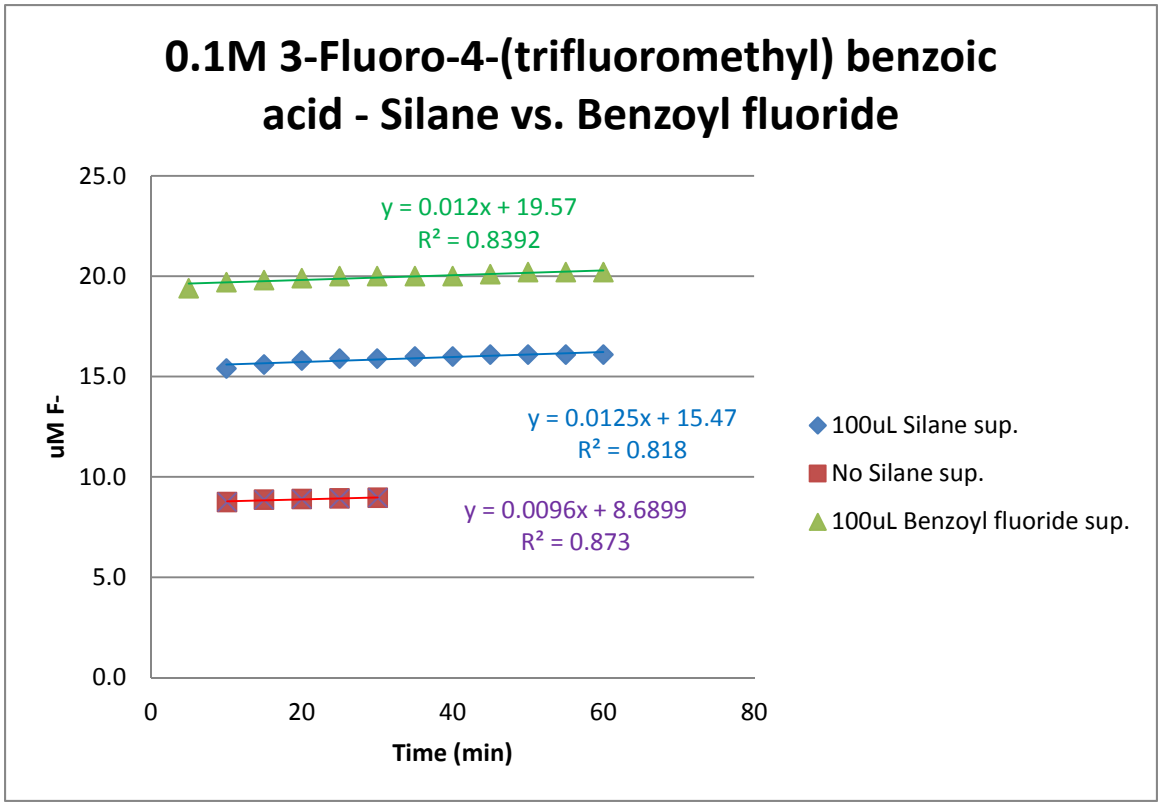


Figure 1. Typical defluorination assay results.

Table 2. Defluorination Rates* of some Organofluorine Compounds

Compound	Hydrolysis Rate (nM F-/min)
Chloropentafluorobenzene	1.0
2,2,2-Trifluoroethanol	0.14
4-Fluorophenol	4.6
Perfluoro-1-octanesulfonyl fluoride	90
2,2-Difluoroethanol	0.4
2-Fluoroanaline	4
2,6-Dichloro-4-(trifluoromethyl) pyridine	6.0
Benzoyl fluoride	980,000
2-Fluoro-6-(trifluoromethyl) benzoyl chloride	4.0
3-Chloro-4-fluoro-5-nitrobenzotrifluoride	16
3-Fluoro-4-(trifluoromethyl) benzoic acid	3.0
2-Fluoro-3-(trifluoromethyl) benzoic acid	3.3
5-Fluoro-2-(trifluoromethyl) benzoic acid	2.0
2-Fluoro-5-nitrobenzotrifluoride	3.0
2-Fluoro-6-(trifluoromethyl) benzonitrile	4.0

*Rates are composite for those compounds with more than one fluoride leaving group.

While most of the organofluorine compounds exhibited very slow hydrolysis, one compound (benzoyl fluoride) hydrolyzed approximately 6 orders of magnitude faster than the others (Figure 2).

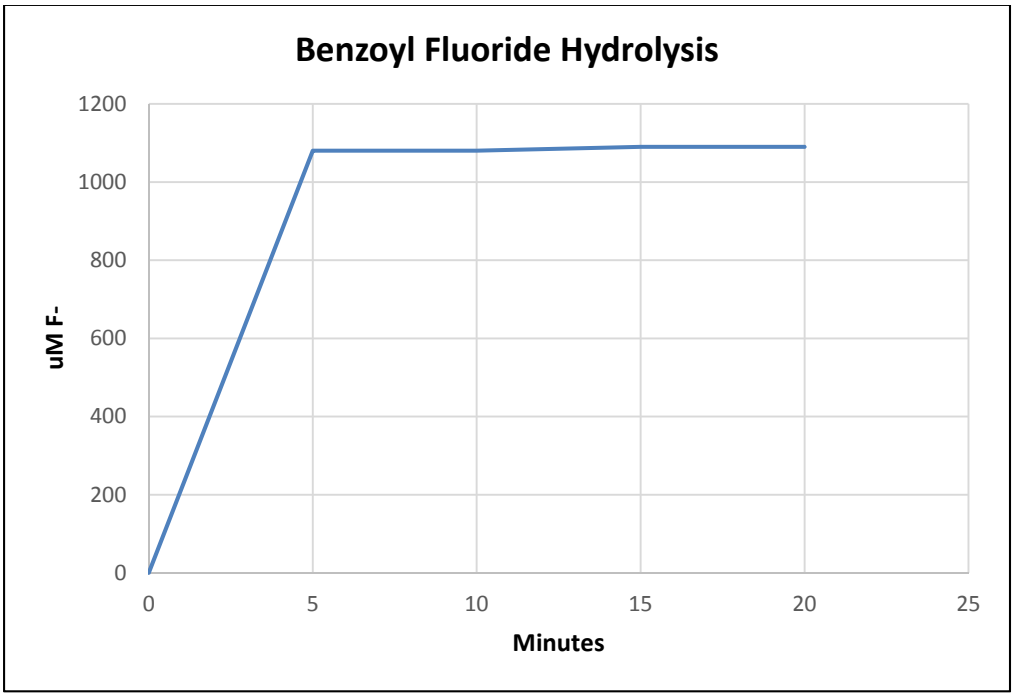
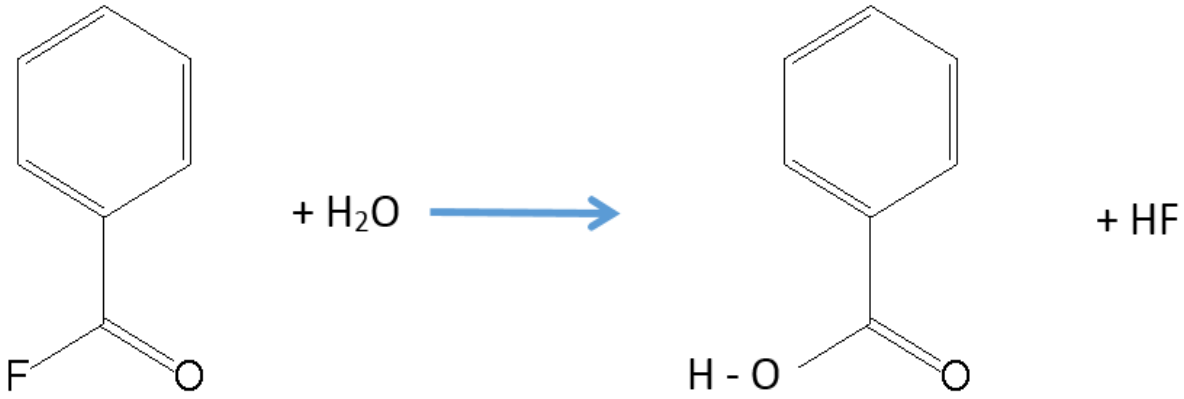


Figure 2. Defluorination of benzoyl fluoride. Assayed in 50 mM BTP with pH 7 at room temperature.

The primary product of the reaction was benzoic acid. The reaction for benzoic acid is depicted below.



Scheme. Benzoic acid reaction.

Accordingly, the cultures that were islolated with benzoyl fluoride as the sole carbon source probably contained benzoic acid.

3.2 Continuous-Loop UV Mutagenesis Reactor

A continuous-loop UV mutagenesis reactor was used to pursue a parallel approach to the isolation of organofluorine-degrading bacteria (Figure 3). This reactor is essentially a chemostat that is used for the purpose of continuously mutagenizing and selecting the organic compounds provided as sole carbon sources.[6]

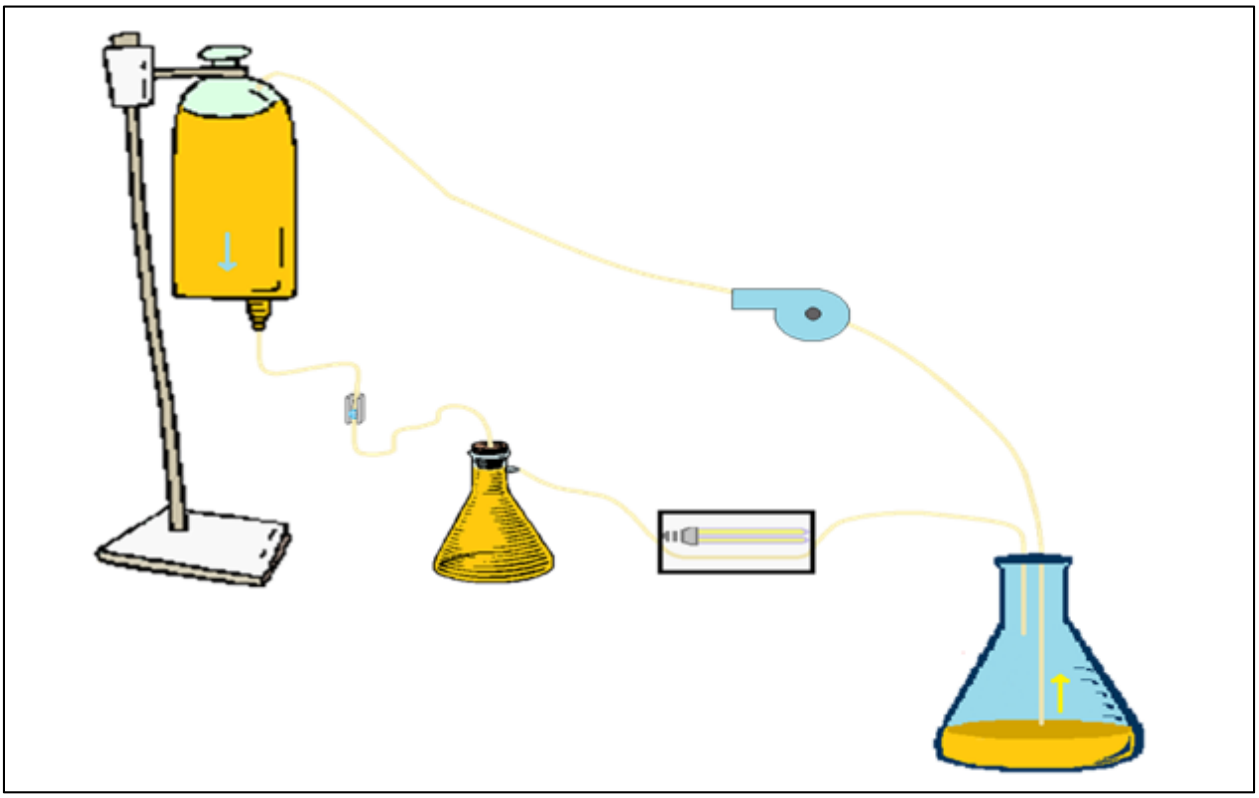


Figure 3. Continuous-loop UV mutagenesis reactor. Environmental isolates are gravity-fed from a drip bag to a continuously stirred Erlenmeyer flask and overflowed into a UV mutagenesis chamber calibrated to kill 90–99% of cells. The overflow from the UV mutagenesis is cycled back to the beginning of the process. Growth (turbidity) can be detected visually in the Erlenmeyer flask.

In an effort to maximize simplicity, the reactor was fed from a drip bag and the mutagenesis was calibrated to the drip rate from the bag. Using a gravity-fed system avoids the possibility of spills related to clogging and pressure build-up, which are common to pump-fed systems. To produce useful mutants, we calibrated the flow through the UV light source to produce an approximately 90–99% kill rate.

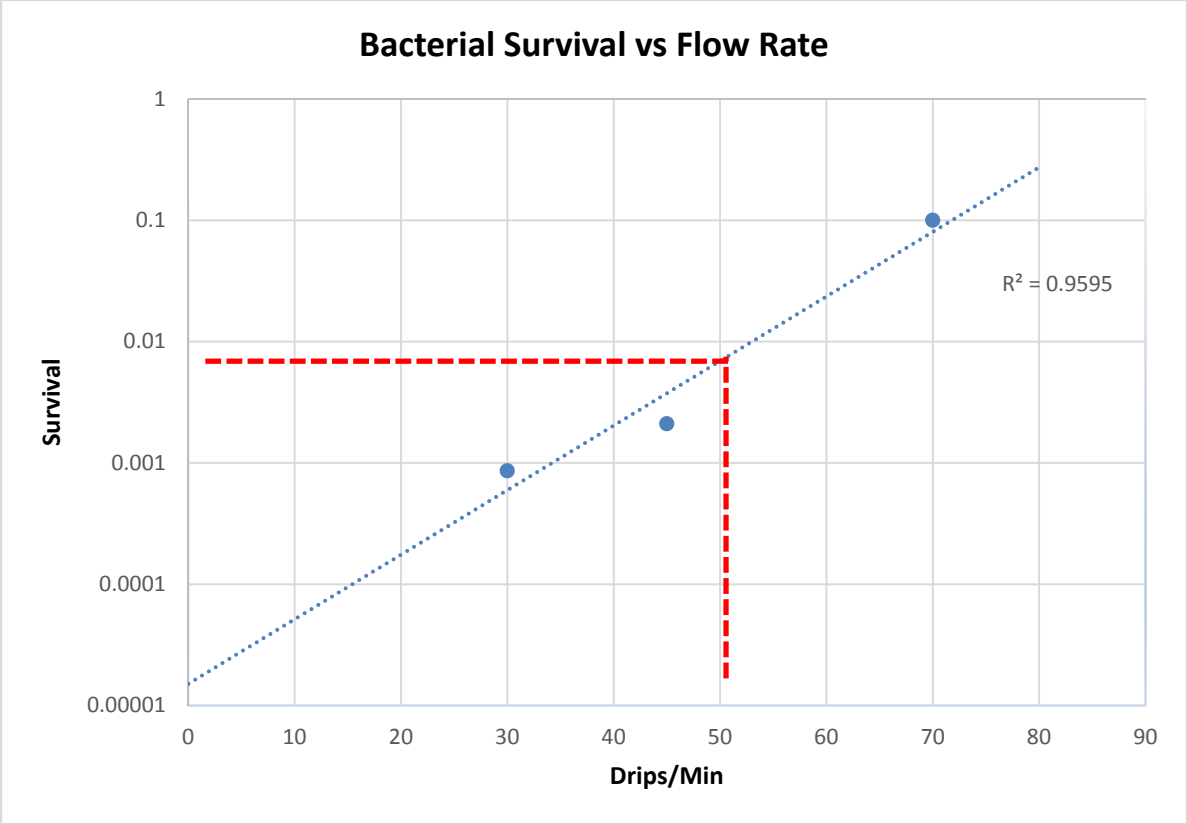


Figure 4. Calibration of mutagenesis reaction. A drip rate of approximately 50 drips/min corresponded to a kill rate of 90–99%. This rate was confirmed with 5 subsequent tests, which yielded kill rates between 96.63 and 97.83%.

Using the calibrated flow rate of 50 drips/min, the mutagenesis reactor was operated continuously for 1 week using 10 mM concentrations of each of the compounds listed in Table 1 (Figure 4). The recycled effluent from that run was contained in a large flask and monitored for any increase in turbidity.

4. CONCLUSIONS

The chemical hydrolysis data will be used to select compounds for future use in the chemostat. The primary criterion for an enrichment substrate is that the compound must be adequately stable (i.e., relatively little hydrolysis should occur over a period of days to weeks). With the exception of benzoyl fluoride, all the other compounds that were tested met that criterion.

Further efforts will be made to identify single-colony isolates, which are capable of using the organofluorine compounds as sole carbon sources for growth.

LITERATURE CITED

1. Key, B.D.; Howell, R.D.; Criddle C.S. Defluorination of Organofluorine Sulfur Compounds by Pseudomonas Sp. Strain D2. *Environ. Sci. Technol.* **1998**, *32*, 2283–2287.

2. Chan, P.W.Y.; Yakunin, A.F.; Edwards, E.A.; Pai, E.F. Mapping the Reaction Coordinates of Enzymatic Defluorination. *J. Am. Chem. Soc*. **2011**, *133* (19), 7461–7468.

3. Barnett, J.E.G.; Jarvis, W.T.S.; Munday, K.A. Enzymatic Hydrolysis of the Carbon-Fluorine Bond of α-D-Glucosyl Fluoride by Rat intestinal Mucosa: Localization of Intestinal Maltase. *Biochemical Journal* **1967**, *103* (3), 699–704.

4. Goldman, P.; Milne, G.W.A. Carbon-Fluorine Bond Cleavage: II. Studies on the Mechanism of the Defluorination of Fluoroacetate. *J. Biol. Chem.* **1966**, *241*, 5557–5559.

5. O'Hagan, D.; Schaffrath, C.; Cobb, S.L.; Hamilton, J.T.G.; Murphy, C.D. Biochemistry: Biosynthesis of an Organofluorine Molecule. *Nature* **2002**, *416*, 279.

6. Fussmann, G. Chemostats: Eco-Evolutionary Dynamics in Chemostats. McGill University Web. http://biology/mcgill.ca/faculty/fussmann/chemostats.html (accessed 3 February 2016).

Blank

DISTRIBUTION LIST

The following individuals and organizations were provided with one Adobe portable document format (pdf) electronic version of this report:

U.S. Army Edgewood Chemical
Biological Center
RDCB-DRB-C
ATTN: Dixon, M.
Gardner, W.

Defense Threat Reduction Agency
DTRA/RD-CBD T
ATTN: Ward, T.
J9-CBS
ATTN: Moore, E.

Department of Homeland Security
DHS ORD CSAC
ATTN: Famini, G.

G-3 History Office
U.S. Army RDECOM
ATTN: Smart, J.

ECBC Technical Library
RDCB-DRB-BL
ATTN: Foppiano, S.
Stein, J.

Office of the Chief Counsel
AMSRD-CC
ATTN: Upchurch, V.

Defense Technical Information Center
ATTN: DTIC OA

ECBC Rock Island
RDCB-DES
ATTN: Lee, K.


EDGEWOOD CHEMICAL BIOLOGICAL CENTER
A U.S. Army RDECOM Laboratory